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13. Abstract *(Maximum 200 Words)* *(abstract should contain no proprietary or confidential information)*

Purpose of the study is to determine effectiveness of novel anticonvulsant and neuroprotectant drugs in lithium-pilocarpine (Li-pilo) induced status epilepticus (SE) used as a model of cholinergic convulsions induced by organophosphorus nerve agents. D-Cycloserine doses of 125 and 250 mg/kg (IP) administered immediately following pilocarpine administration or after 5 min of SE induced no anticonvulsant or neuroprotective effect. Preliminary experiments with 200 mg/kg ACPC (1-aminocyclopropanecarboxylic acid) indicate significant neuroprotection in the absence of anticonvulsant activity in Li-pilo SE. In a study outside the scope of the project, the anesthetic propofol inhibited SE activity and induced significant neuroprotection when administered in multiple doses. Further investigation of propofol as a medical countermeasure to nerve agents is warranted. Additional studies with hydroethidine have characterized reactive oxygen species production in Li-pilo SE.

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pilocarpine, cycloserine, propofol, hydroethidine

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INTRODUCTION

The project will test novel treatment strategies for the N-methyl-D-aspartic acid (NMDA) component of organophosphorous (OP) nerve agents that are intended to complement or improve current treatments. OP agents induce an initial cholinergic over stimulation that is followed by a glutamatergic over stimulation of NMDA receptors. The excessive NMDA activation and excitotoxicity results in status epilepticus (SE), neurological deficit and neuropathology associated with OP intoxication. The lithium-pilocarpine (Li-pilo) model of cholinergic convulsions in rats will be used as the experimental model of OP nerve agent-induced SE. The project will test compounds that act as anticonvulsants in that they inhibit the seizures by acting on the strychnine-insensitive glycine site of the NMDA receptor. The project will also test neuroprotectants that protect the brain from seizure-induced neuropathology by interfering with NMDA receptor-mediated intracellular excitotoxicity mechanisms. Experimental parameters that will be used to assess the test drug activity are: 1) electrocorticograph (ECoG) determination of continuous high amplitude spiking as a measure of SE duration; 2) spontaneous activity before and after SE as a measure of neurological deficit; 3) neuropathology in brain regions damaged by Li-pilo SE.

The initial compound to be tested in this project was D-cycloserine (DCS) which is the principle topic of this report. DCS is a partial agonist of the strychnine-insensitive glycine site on the NMDA receptor that has 70% the efficacy of glycine (Henderson et al., 1990; Hood et al., 1989; Watson et al., 1998). As for all partial agonists, DCS is a high efficacy agonist by itself, but in the presence of a full agonist (glycine) acts as an antagonist. It was hypothesized that during SE, DCS would antagonize the activity of endogenous glycine at the strychnine-

insensitive sites and exert anticonvulsant activity. This hypothesis is supported by evidence that DCS induces anticonvulsant activity in maximal electroshock seizures (Peterson, 1992; Peterson and Schwade, 1993; Walz, 1998) and kindled amygdaloid seizures (Loscher et al., 1994; Rundfeldt et al., 1994). DCS also produces impressive reductions in the behavioral seizures associated with kainate-induced SE (Baran et al., 1994). In addition, DCS exerts nonselective NMDA receptor antagonism (Baron et al., 1992; Monahan et al., 1989; Baxter and Lanthorn, 1995) which would provide additional anticonvulsant activity against the NMDA component of cholinergic convulsions.

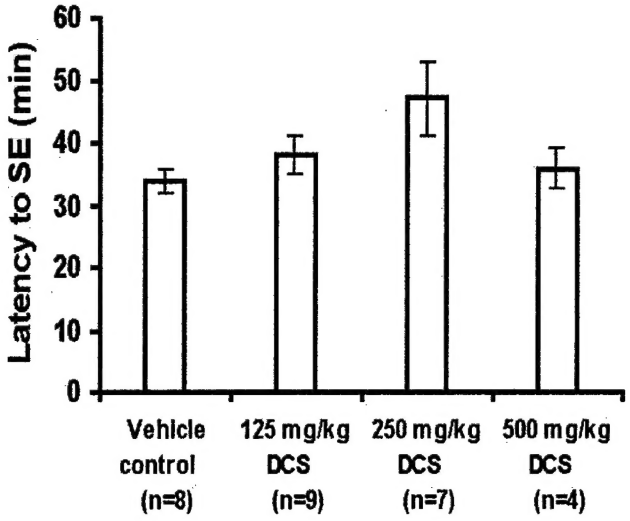
BODY

Tasks Completed from the Approved Statement of Work

The purpose of the study was to use the Li-pilo model of SE in rats to test the anticonvulsant activity of D-cycloserine (DCS) against the NMDA component of cholinergic convulsions. Li administration (3 mmol/kg, SQ) followed 20-24 hours later by pilocarpine (25 mg/kg, SQ) induces a SE of 2-3 hours duration that serves as a model of the convulsions induced by OP nerve agents. SE-induced afterdischarge was defined as the duration of continuous high amplitude ECoG spiking. Propofol (55 mg/kg, i.p.) was administered 3 hours following SE onset. During the first year of this study it was determined that propofol significantly enhanced 24 hour survival without affecting the neuropathology induced by 3 hours of SE. (A full report of the propofol study is provided in the section "Completed tasks Outside the Approved Statement of Work"). The test compounds were to be administered either immediately following (within 1 minute) the pilocarpine administration (exposure treatment) or 5 minutes following the onset of SE (SE treatment). The objectives include testing saline as the vehicle control treatment for both the exposure and SE treatments. This was to be followed by a dose-response study of the novel anticonvulsant D-cycloserine (Peterson, 1992; Peterson and Schwade, 1993), a partial agonist of the strychnine-insensitive glycine receptor on the NMDA receptor-ionophore complex (Henderson et al., 1990; Hood et al., 1989; Watson et al., 1990).

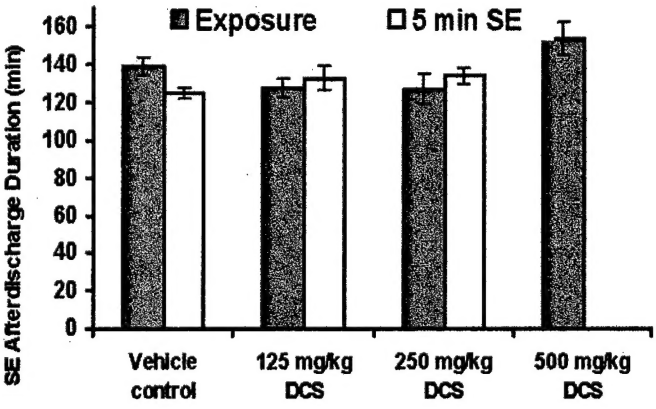
DCS doses of 250 and 500 mg/kg were originally proposed for study. The 250 mg/kg DCS dose was tested as originally planned and the results are presented below. However, a preliminary study with 4 rats determined that the 500 mg/kg DCS exposure treatment appeared to exacerbate the SE. While the latency to SE onset was not reduced (Figure 1), the duration of the SE afterdischarge was significantly increased by 500 mg/kg DCS (Figure 2) and the gross neuropathological damage observable in whole brain sections was enhanced (Figure 5). An "inverted U" dose response curve for the anticonvulsant activity of DCS in kainate-induced SE has been reported (Baran et al., 1994) as 320 mg/kg provided less anticonvulsant activity than 160 mg/kg. In light of such data it was determined that 125 mg/kg DCS would be the second DCS dose tested in the Li-pilo-induced SE instead of the 500 mg/kg dose. The 125 mg/kg dose was also within the 80-160 mg/kg range reported by Baran et al. (1994) to be effective in kainate SE.

Figure 1. Latency to SE onset was the time from pilocarpine administration to the start of SE as defined by continuous high amplitude ECoG spiking. DCS administered immediately following pilocarpine (exposure treatment) had no significant effect on SE latency (one way ANOVA).



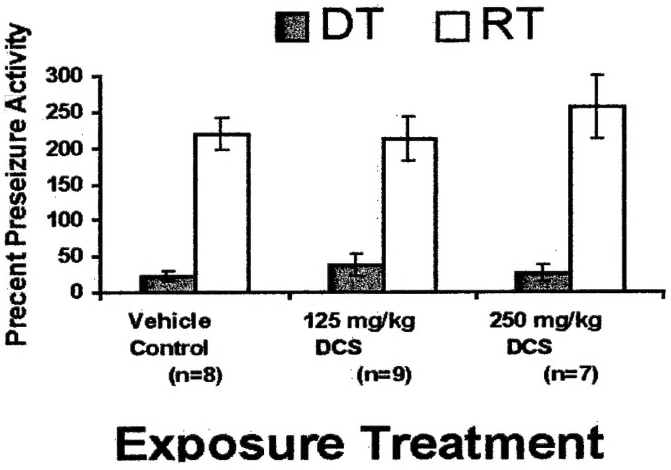
DCS had no anticonvulsant or neuroprotectant activity in Li-pilo-induced convulsions. The 125 and 250 mg/kg doses administered immediately following pilocarpine (exposure treatment) had no effect on the latency to SE onset (Figure 1). DCS had no significant effect on SE afterdischarge duration whether administered as exposure treatment or following 5 min of SE (Figure 2).

Figure 2. SE afterdischarge duration was defined as the period of continuous high amplitude ECoG spiking. Administration of 125 or 250 mg/kg DCS either immediately following pilocarpine (exposure) or after 5 minutes of SE had no significant effect on SE afterdischarge duration (one way ANOVA). 500 mg/kg DCS (n=4) administered as exposure treatment significantly increased AD duration as compared to vehicle control (t-test).



Spontaneous activity was monitored as a measure of the neurological deficit induced by SE and the possible neuroprotection that might be induced by DCS. Spontaneous activity was monitored for 10 minutes in an activity monitor the day of the lithium administration (preseizure) and again 24 hours following pilocarpine administration. In vehicle control animals, the Li-pilo SE produced a decrease in distance traveled (DT) and an increase in resting time (RT) indicating a neurological deficit (Figures 3 and 4) (Lallement et al., 1997; Lallement et al., 1998; Walton and Treiman, 1991). DCS treatment either at exposure or following 5 minutes SE had no effect on the SE-induced neurological deficit (one way ANOVA) (Figures 3 and 4).

Figure 3. Effects of Li-pilo SE on spontaneous activity expressed as a percent of preseizure activity. Spontaneous activity was determined for 10 minutes in an activity monitor. Distance traveled (DT) was decreased and resting time (RT) increased indicating a SE-induced decrease in spontaneous activity. Exposure treatment had no effect on the neurological deficit.



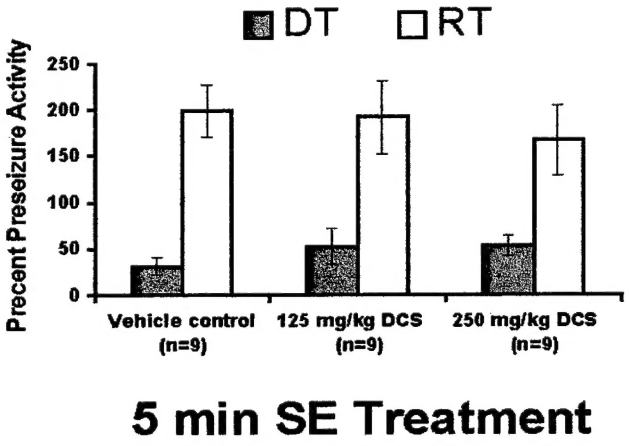


Figure 4. DCS administration following 5 minutes of Li-pilo-induced SE (SE treatment) also had no effect on neurological deficit as measured by distance traveled (DT) and resting time (RT).

Twenty-four hours following pilocarpine administration and immediately following spontaneous activity assessment the animals were sacrificed and perfusion fixed for histological anaylsis of SE-induce neuronal damage. The 24 hour survival period is optimal for the demonstration of neuropathological damage in both Li-pilo (Clifford et al., 1987; Fujikawa, 1996) and soman SE (McDonough et al., 1998). Formalin-fixed brains were removed and embedded in paraffin for sectioning. A 5 μM section was taken every 150 μM through the brain tissue 0.8 to 4.8 mm posterior to bregma. This area was chosen for the histopathological analysis for efficiency and because it contains critical brain nuclei that exhibit the greatest degree of damage from soman (McDonough et al., 1998) and Li-pilo convulsions (Clifford et al., 1987; Motte et al., 1998; Fujikawa et al., 1999; Peredery et al., 2000). The sections were prepared with hematoxylin and eosin (H&E) staining and sent to the coinvestigator, Dr. James Griffith of the Penn State College of Medicine, for pathological analysis. A scale of lesion severity developed for assessing soman toxicity (McDonough et al., 1989) was used to score the neuronal damage in

a blinded fashion. The scale is as follows: 0=no damage; 1=5% necrotic or malacic tissue; 2=6-15%; 3=16-40%, 4=> 40%. Statistical comparisons were made by Kruskal-Wallis H-test.

Li-pilo-induced convulsions of 3 hours duration prior to propofol administration produced a significant degree of neuropathology in the piriform and perirhinal cortex. As shown in Figure 5, a loss of tissue was clearly visible in these critical regions of H&E stained sections even without magnification. This limbic cortex damage did not appear to be altered by 125 or 250 mg/kg DCS. However, following 500 mg/kg DCS the neuronal damage extended into the parietal cortex regions and the piriform tissue was sufficiently malacic that the section tattered. As a result of this observation the 500 mg/kg DCS dose was not tested further.

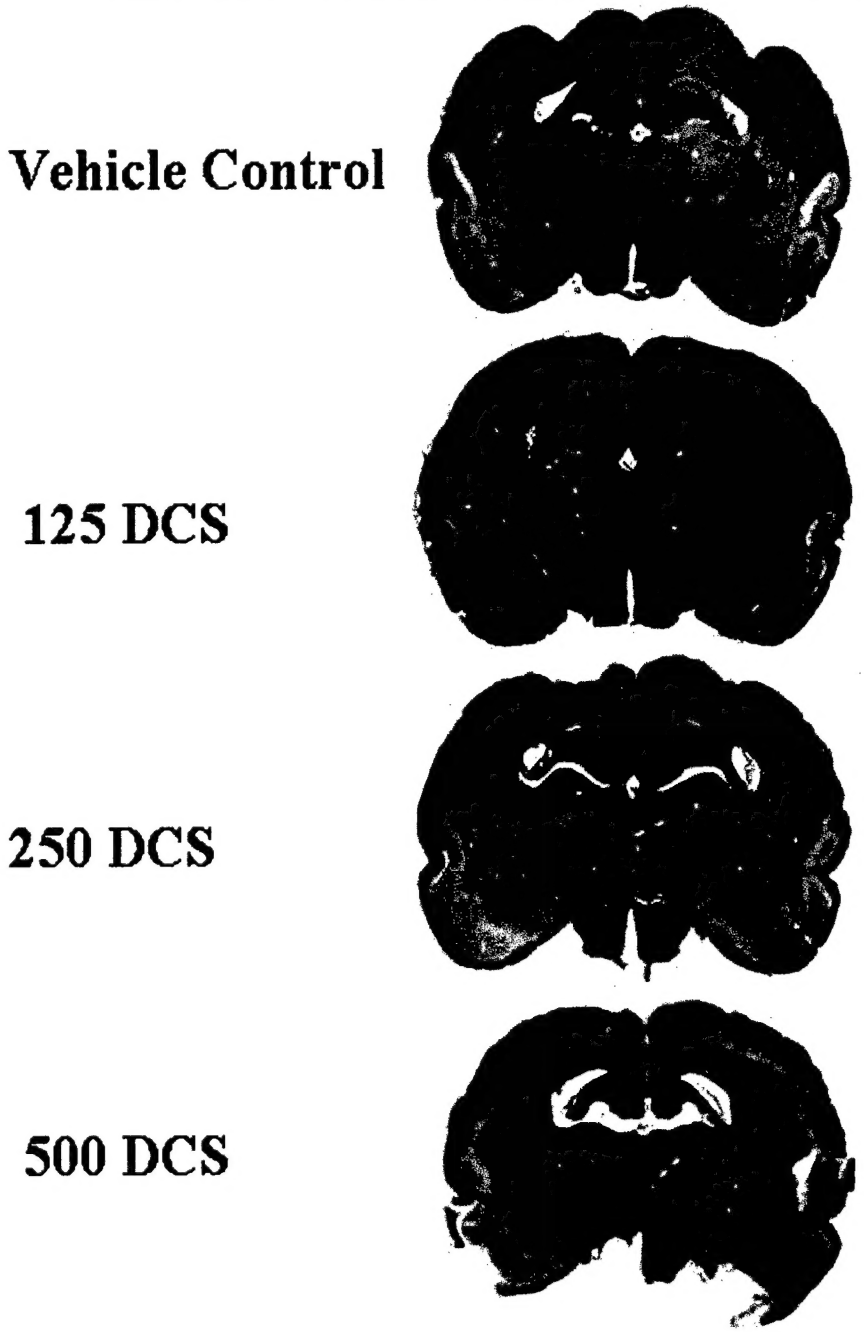


Figure 5. Representative H&E sections of rat brains demonstrating the extent of Li-pilo-induced neuropathology. Vehicle control, 125 and 250 mg/kg doses were administered 5 minutes following SE onset (SE treatment). The 500 mg/kg dose was administered immediately following pilocarpine (exposure treatment). The lower DCS doses had no effect on the SE-induced neuropathology in the piriform and perirhinal cortical regions, while the 500 mg/kg dose apparently exacerbated the neural damage as the malacic tissue extended into the parietal cortex and the piriform cortex did not maintain morphological integrity during sectioning.

As indicated in Table 1, the brain structures in the vehicle control group demonstrated mean neural damage scores of 1.5 to 4.0 corresponding to greater than 16-40% necrotic or malacic tissue. These data indicate that the Li-pilo model of OP nerve agent SE in our hands induces a quantifiable degree of neuronal damage in the expected brain structures (Clifford et al., 1987; McDonough et al., 1989; McDonough et al., 1998; Fujikawa et al., 1999). In addition, this neural damage was comparable to or in excess of that reported for the same brain structures in a rat model of soman-induced convulsions (McDonough et al., 1989; McDonough et al., 1998). Treatment with DCS either at exposure or following 5 min SE had no significant effect on the Li-pilo-SE induced neuronal damage in most brain regions (Table 1). The exceptions were the mediodorsal (MD) thalamic nucleus and the pretectal nucleus (APTD) in the 125 mg/kg DCS exposure treatment groups in which the neuropathology was significantly greater than the vehicle control group (Kruskal-Wallis H-test, $P<0.02$).

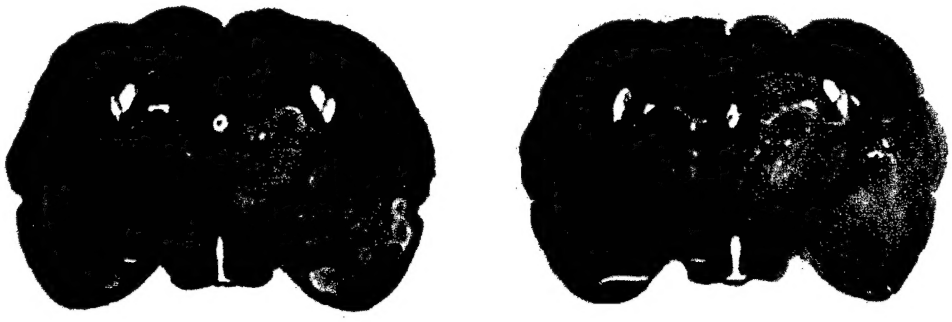
Table 1. Mean neural damage scores for brain structures in the region –0.8 to –4.8 mm from bregma. DCS had no effect to reduce Li-pilo-induced neuronal damage. * indicates significant difference ($P< 0.02$) from corresponding vehicle control group.

Brain Region	Exposure treatment			5 minute SE		
	control(n=8)	125 DCS(n=9)	250 DCS(n=7)	control(n=9)	125 DCS(n=9)	250 DCS(n=9)
parietal cortex (Par1)	2.65	2.92	2.95	3.00	2.54	2.54
occipital cortex (Oc)	2.69	2.54	2.59	2.80	2.44	2.40
perirhinal cortex (PRh)	3.69	3.74	3.61	3.59	3.16	3.42
piriform cortex (Pir)	4.00	4.00	4.00	3.97	3.83	3.98
cortical amygdala (Co)	3.91	3.83	3.68	4.00	3.97	3.94
medial amygdala (BM)	3.16	3.26	3.31	3.44	3.44	3.43
lateral amygdala (La)	3.58	3.89	3.85	3.76	3.73	3.81
mediodorsal thalamus (MD)	2.25	2.93 *	2.60	3.09	2.94	2.89
lateraldorsal thalamus (LD)	2.25	2.87	2.58	3.20	3.05	3.12
pretectal nucleus (APTD)	2.13	2.99 *	2.39	2.74	2.79	2.66
CA3	2.59	2.66	2.41	2.81	2.79	2.72
CA1	1.34	1.27	1.03	1.46	1.24	1.28
amygdalopiriform (Apir)	4.00	4.00	4.00	4.00	3.97	4.00

DCS induced no anticonvulsant or neuroprotective activity in Li-pilo-induced convulsions. No significant differences between vehicle control and DCS treatment groups were found for any of the parameters tested including latency to SE onset, duration of SE, neurological deficit or neuronal damage. This was an unexpected result considering the hypothesized role of NMDA receptors in cholinergic convulsions (Solberg and Belkin, 1997; McDonough and Shih, 1997) and the activity of DCS both as a partial agonist at the strychnine-insensitive glycine site (Henderson et al., 1990; Hood et al., 1989; Watson et al., 1998) as well as an NMDA receptor antagonism (Baron et al., 1992; Monahan et al., 1989; Baxter and Lanthorn, 1995). DCS is also an active anticonvulsant in standard experimental models of epilepsy (Peterson, 1992; Peterson and Schwade, 1993; Walz, 1998; Loscher et al., 1994; Rundfeldt et al., 1994).

In contrast to the lack of activity in Li-pilo convulsions, DCS has been reported to reduce the behavioral convulsions associated with kainate-induced SE (Baran et al., 1994). In that study, DCS induced a dose-related decrease in kainate behavioral convulsions at the 80 and 160 mg/kg doses (i.p.) (Baran et al., 1994). At 320 mg/kg the anticonvulsant activity was not as great as at 160 mg/kg dose, suggesting proconvulsant activity at higher doses or an "inverted U" dose response curve. In the present study a proconvulsant effect of DCS was also observed at higher doses as 500 mg/kg enhanced the SE afterdischarge duration and appeared to enhance the SE evoked neuropathology. However, even the DCS dose (125 mg/kg) which is in the 80-160 mg/kg range that is reported to be anticonvulsant in kainate SE was ineffective in Li-pilo convulsions. This discrepancy may result from the present study evaluating multiple parameters of SE activity including latency to SE, SE duration, neurological deficit and neuropathology while the kainate study evaluated only behavioral convulsions (Baran et al., 1994). Perhaps an in-depth study of additional SE parameters would reveal less DCS anticonvulsant activity in kainate seizures. Alternatively, given the difference in the neuronal mechanisms between kainate and Li-pilo SE (Goodman, 1998) it is conceivable that a population of NMDA receptors that are more responsive to DCS are involved in kainate SE.

Initial experiments have begun with ACPC, the next compound to be tested in this project. ACPC (1-aminocyclopropanecarboxylic acid) is also a high efficacy partial agonist of the strychnine-insensitive glycine receptor (Marvizon et al., 1986) that has anticonvulsant activity in NMDA-induced seizures in mice (Skolnick et al., 1989; Bisaga et al., 1993) and seizures in genetically epilepsy-prone rats (Smith et al., 1993). A 200 mg/kg exposure treatment dose has been tested in 5 rats and appears to reduce the Li-pilo-induced neuropathology. Although the blinded histopathological analysis has not been performed, observation of whole H&E stained sections reveals markedly reduced neuropathology in the piriform and perirhinal cortex (Figure 6). Interestingly, ACPC does not appear to reduce the SE afterdischarge duration (data not shown). Although preliminary, this data suggests ACPC induces neuroprotection without anticonvulsant activity. Neuroprotective activity has been reported for ACPC in that it inhibits glutamate-induced cell damage in cerebellar granule cell cultures (Fossom et al., 1995a; Boje et al., 1993) and protects against ischemic cell damage in an experimental model of ischemic stroke (Fossom et al., 1995b; Von Lubitz et al., 1992). Given the common action of ACPC and DCS at NMDA receptors, failure of DCS to alter Li-pilo-induced SE suggests partial agonist activity at the strychnine-insensitive glycine receptor is not a mechanism of ACPC neuroprotection.



Vehicle Control **200 mg/kg ACPC**

Figure 6. Representative H&E sections of rat brains demonstrating the extent of Li-pilo-induced neuropathology and the neuroprotection induced by ACPC. The neural damage in piriform and perirhinal cortex appears to be greatly reduced following ACPC exposure treatment.

Deviations from the original experimental protocol have been minor. As indicated above, 125 mg/kg DCS was tested instead of 500 mg/kg as originally proposed because 500 mg/kg clearly exacerbated the seizures. In addition, 55 mg/kg propofol i.p. is now administered 3 hours following the onset of electrographically defined SE. This enhances the 24-hour survival rate to nearly 100% without affecting the extensive neuropathology induced by the 3 hours of Li-pilo convulsions. Mefenamic acid was originally slated to be the next compound tested. This was changed to ACPC so that a manuscript resulting from the DCS and ACPC studies (both partial agonists of the strychnine-insensitive site) could be prepared within the next 6 month reporting period.

Completed Tasks Outside of the Approved Statement of Work

The nonbarbiturate anesthetic propofol had been developed as a treatment to enhance 24 hour survival. That data is being prepared in manuscript form and is expected to be submitted for publication in February, 2003. Propofol had a significant impact on survival following 3 hours of Li-pilo-induced convulsions. All rats treated with 55 (n=3) or 65 (n=3) mg/kg propofol i.p. following 3 hours of SE survived the 24 hour period following pilocarpine administration (Fig. 7). In contrast, only 3 of 6 animals survived the 24 hour period when administered 50 mg/kg propofol.

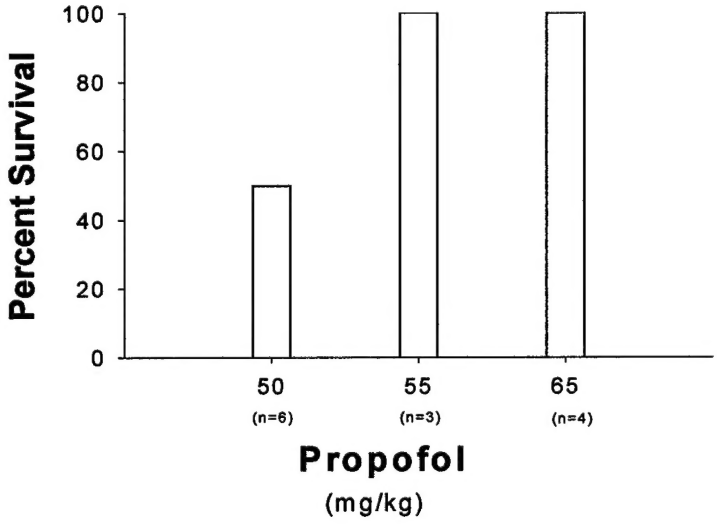


Figure 7. Propofol induced a dose-dependent increase in 24 hour survival following Li-pilo SE. Propofol was administered i.p. 3 hours after the onset of SE as defined by continuous high amplitude ECoG spiking.

Propofol completely inhibited all ECoG spiking activity when administered 3 hours after onset of SE (Fig. 8). Propofol induced a complete suppression of ECoG spiking at which time the animals appeared anesthetized. Twenty-four hours following pilocarpine administration the rats were ambulatory and exhibited interictal ECoG spiking (Fig. 8).

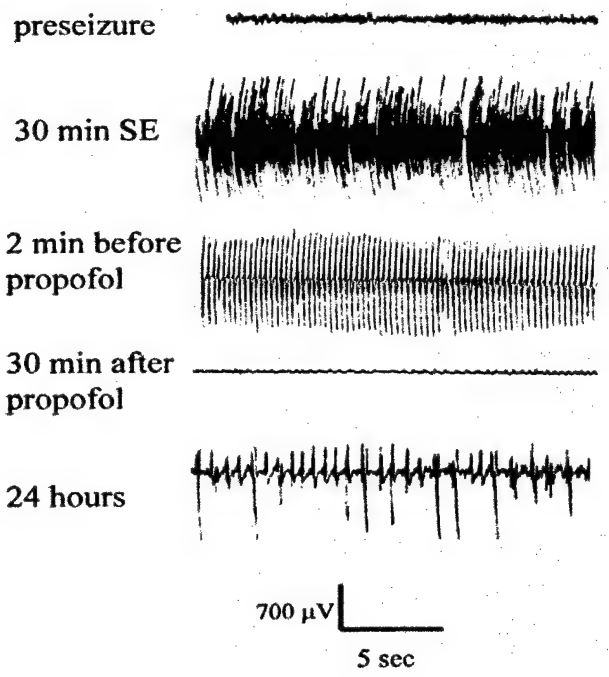


Figure 8. Representative ECoG tracings from a rat in Li-pilo-induced SE that was treated with 55 mg/kg propofol. Propofol was administered 3 hours after the onset of SE as defined by continuous high amplitude ECoG spiking. An example of ECoG activity representative of SE is shown in the tracing labeled "30 min SE". Propofol suppressed all ECoG spiking as shown in the tracing labeled "30 min after propofol". Rats appeared anesthetized when the ECoG spiking was suppressed. Interictal spiking was present 24 hours following pilocarpine administration as seen in the bottom tracing.

Propofol administered following 5 minutes of Li-pilo SE inhibited all ongoing seizure activity. The complete suppression of ECoG spiking required an average of 12.2 minutes (n=5) which was significantly less (t-test, $P < 0.05$) than the 20.8 minutes required following 3 hour SE (20.8 min). The rats appeared anesthetized when ECoG spiking was arrested. Propofol administered immediately following pilocarpine (exposure treatment) also induced an anesthetized state and prevented the onset of all seizure activity (n=5).

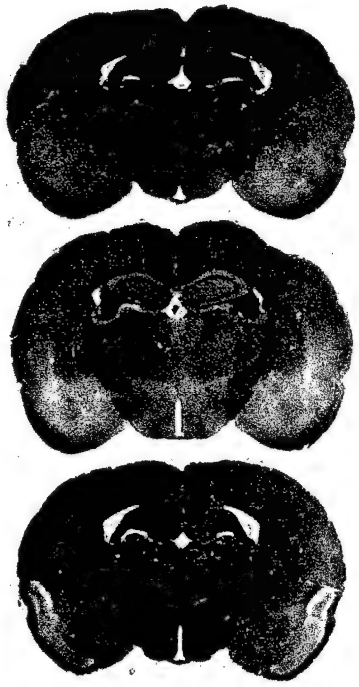


Figure 9. Representative H&E sections demonstrating the extent of Li-pilo-induced neuropathology and the neuroprotection induced by propofol.

The 55 mg/kg propofol dose administered 5 minutes following the onset of SE provided significant neuroprotection. As shown by the sections in Fig 9, these brains appeared indistinguishable from control animals. Histopathological scores were not different from animals without SE (Fig. 10A). Similarly, the neuropathology of rats treated with 55 mg/kg propofol immediately following pilocarpine (exposure treatment) did not differ significantly from the seizure control animals (No SE) as shown in Fig 10A.

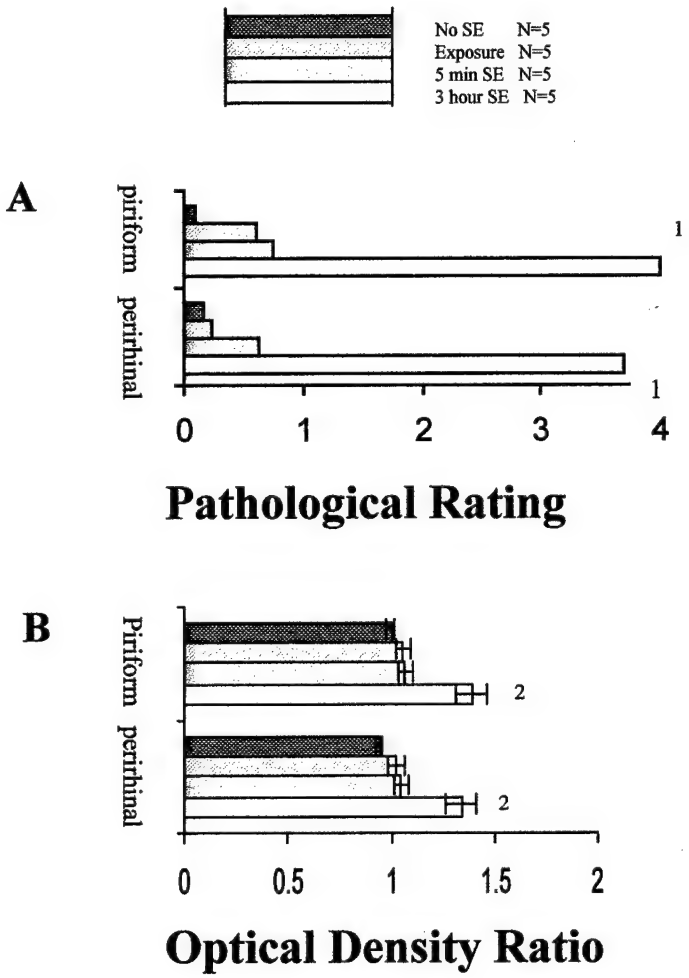


Figure 10. Histopathological and optical density analysis of Li-pilo-SE-induced neuronal damage in the piriform and perirhinal cortex. **A.** Using histopathological ratings the neuronal damage in the 3 hour SE group was significantly greater than that in the No SE, exposure and 5 min SE group (1, $P<0.05$, Mann-Whitney U-Test). **B.** Using optical density ratio the neuronal damage in the 3 hour SE group was significantly greater than that of all other groups (2, $P<0.05$, ANOVA and Newman-Kuels post hoc test.

The 55 mg/kg propofol dose administered 3 hours after the onset of SE did not provide neuroprotection from the Li-pilo-induced convulsions. As shown in Fig. 9, neuronal damage was clearly visible in the piriform and perirhinal cortices. The histopathological scores were significantly greater (Mann-Whitney U-test, $P<0.05$) in the rats with 3 hours SE as compared to the rats with no SE, exposure treatment or 5 minutes SE treatment (Fig. 10A). The 55 mg/kg propofol treatment improves 24 hour survival to 100% but allows expression of the SE-induced neuropathology. Allowing 3 hours of SE was necessary for this study as the duration of the cortical afterdischarge defined SE is one of the seizure parameters measured in this project.

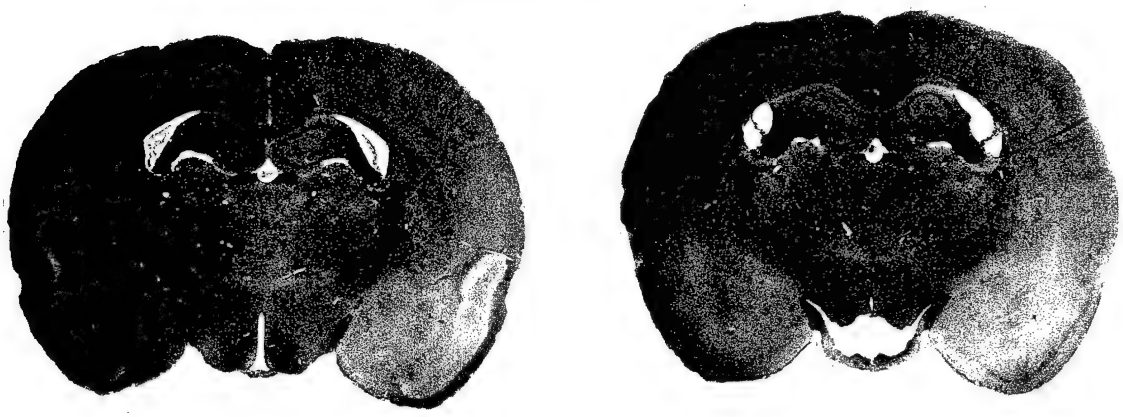
Three hours of Li-pilo-induced seizures is also sufficient to induce significant neuronal necrosis (Fujikawa et al., 2000). In addition, 3 hours seizure activity followed by 24 hours recovery is associated with maximal neuronal damage in both Li-pilo (Fujikawa et al., 1999) and pilocarpine seizures (Fujikawa, 1996).

We have developed a novel optical density analysis method for quantifying the severe malacic damage created in the piriform and perirhinal areas. The optical density analysis was performed using H&E stained sections taken from the same sections used for histopathological evaluation. Images were acquired using a stereomicroscope and a digital camera. An empirical parameter termed optical density was used as a measurement of necrotic and malacic tissue damage in piriform and perirhinal cortex. The total light intensity in electronically defined areas of equal size in both the piriform and perirhinal cortex was determined from digital images of the brain sections using the luminosity histogram function of the Adobe Photoshop software. This was compared by ratio to the luminosity of identical electronically defined regions within the hypothalamus of the same section. Because the hypothalamus receives little or no damage in Li-pilo convulsions (Clifford et al., 1987; Motte et al., 1998), the optical density in rats without SE was approximately 1 as the light intensity or luminosity in the cortical areas was similar to that in the hypothalamus. In rats with SE the optical density ratio was greater than 1.0 as the necrotic and malacic tissue in the piriform and perirhinal cortex allowed a greater transmission of light.

As shown in Figure 10B, the optical density ratio was significantly greater in the animals that experienced 3 hours SE than all other groups tested ($P<0.05$, ANOVA and Newman-Kuels post hoc test). The optical density rations of these regions approached 1.5 (Fig 10B) indicating 50% increase in light transmission through these severely damaged regions. A preliminary correlation analysis of the histopathological rating and the optical density ratio in the perirhinal and piriform cortex determined r-values of .80 and .87, respectively. We propose that optical density ratio analysis may serve as a means to rapidly quantify the most severe neuropathological damage in the piriform and perirhinal cortical regions and would be useful for experiments screening anticonvulsant or neuroprotectant agents in Li-pilo convulsions.

Although preliminary, initial experiments indicate that repeated propofol doses induce a significant neuroprotectant effect. As shown in Fig. 11, one additional 20 mg/kg propofol dose 2 hours following the initial 55 mg/kg dose prevented the lesions normally seen in the piriform and perirhinal cortices following 3 hours of SE. In Fig 11 the depicted brain sections are both from rats administered 55 mg/kg propofol following 3 hours SE. The only difference is that the section on the right of Fig 11 is from a rat administered an additional 20 mg/kg propofol dose i.p. 2 hours after the initial 55 mg/kg dose. Concluding experiments are being conducted and the histopathological ratings and optical density measurements have yet to be completed. If these additional experiments are consistent it would appear that single doses of propofol are anticonvulsant and can prevent or terminate seizure activity while additional doses are neuroprotective and can prevent the development of brain damage.

3 Hour SE



55 mg/kg propofol

55 mg/kg propofol plus
20 mg/kg propofol

Figure 11. Neuroprotection induced by multiple doses of propofol. The sections above were taken from rats that experienced 3 hours SE followed by 55 mg/kg propofol. The section on the right is from an animal that received 20 mg/kg propofol 2 hours after the initial 55 mg/kg dose. Note the complete absence of the typical piriform and perirhinal neuropathology in the animal that received multiple propofol doses. This preliminary evidence suggests remarkable neuroprotectant activity by propofol.

As indicated in Reportable Outcomes, a publication (Epilepsy Research 49 (2002): 226-238) has been produced that was supported by this project. Although all of the experiments reported in that publication had been completed before this project was funded, funds from this project were used to cover the costs of manuscript preparation. A copy of the manuscript is provided in the Appendix while a brief description is provided here.

The study tested the hypothesis that reactive oxygen species and specifically superoxide is produced in discrete brain regions during Li-pilo-induced SE. The selective oxidation of hydroethidine to ethidium was used as a fluorescent marker of superoxide production. Using fluorescence microscopy and digital imaging it was possible to evaluate superoxide production in specific brain regions during SE. Regions which are damaged by Li-pilo SE and that produced superoxide as indicated by accumulation of ethidium included the parietal cortex, piriform cortex, perirhinal cortex, lateral amygdala, mediodorsal thalamus and laterodorsal thalamus. This suggests that superoxide production may serve as a mechanism of neural injury in these regions. Other regions injured by Li-pilo seizures but that did not demonstrate enhanced superoxide production included the basolateral amygdala and hippocampus. This indicated that SE-induced neuropathology in those regions might not involve superoxide. This study demonstrates a means to quantify superoxide production and may serve as a technique to test hypothesized mechanisms of reactive oxygen species-mediated neural injury in cholinergic convulsions.

KEY RESEARCH ACCOMPLISHMENTS

- Completed study of D-cycloserine.
- Completed all data collection for the SE treatment vehicle control groups.
- Initiated study of ACPC.
- The data from the propofol study has been prepared as an abstract that has been accepted for presentation at the American Epilepsy Society meeting in December 2002. The same data is being prepared in manuscript form for submission to Epilepsy Research in February 2003.

REPORTABLE OUTCOMES

- Peterson, S.L., Purvis, R.S. and Griffith, J.W., (2002). Propofol Inhibition of Lithium-Pilocarpine-Induced Status Epilepticus. Epilepsia 43 (S7): 19.
- Peterson, S.L., Purvis, R.S. and Griffith, J.W., Propofol Inhibition of Lithium-Pilocarpine-Induced Status Epilepticus and Associated Neuropathology in Rats. (Manuscript In Preparation).
- Peterson, S.L., Morrow, D., Liu, S. and Liu, K.J., (2002). Hydroethidine Detection of Superoxide Production During Lithium-Pilocarpine Model of Status Epilepticus. Epilepsy Research 49:226-238.

CONCLUSIONS

The project is on schedule. There are no anticipated complications that will prevent completion of all approved Statement of Work tasks as originally scheduled.

D-Cycloserine (DCS) induced no anticonvulsant or neuroprotectant activity in Li-pilo-induced convulsions. This is in sharp contrast to reports of significant anticonvulsant activity against the behavioral component of kainate-induced SE in rats (Baran et al., 1994). The discrepancy indicates significant differences in the underlying neuronal mechanisms between Li-pilo and kainate convulsions. This finding lends further support to the hypothesis that only cholinergic convulsions are appropriate models of the neuropathology associated with nerve agent exposure.

In contrast, preliminary data from the planned ACPC study indicates ACPC-induced neuroprotection in Li-pilo convulsions. Given the similar action of DCS and ACPC at the NMDA receptor site, these preliminary data would indicate that partial agonist activity at strychnine-insensitive glycine receptors is not the mechanism of action of ACPC. Instead, hypothesized antioxidant properties may be responsible (Fossom et al., 1995a; 1995b; Boje et al., 1993; Von Lubitz et al., 1992). Complete results will be presented in future reports.

The initial development and characterization of propofol as treatment for Li-pilo-induced convulsions is near completion and in preparation as a manuscript. Propofol prevented the onset of Li-pilo convulsions, terminated ongoing SE and enhanced the 24 hour survival of all animals. Rats administered propofol immediately following pilocarpine (Exposure Treatment) failed to

develop seizure activity or neuropathology. Ongoing SE was terminated in rats administered propofol after 5 min of SE (5 min SE) and the observed neuropathology was not significantly greater than the seizure-free control rats. Rats with 3 hour SE prior to propofol exhibited severe neuropathology, but nevertheless survived 24 hours. Rats with 3 hour SE prior to 55 mg/kg propofol followed 2 hours later by an additional 20 mg/kg propofol exhibited markedly reduced neuropathological damage. This data indicates that propofol is an effective anticonvulsant at all stages of Li-pilo-induced SE. Following a slightly higher dose propofol exhibited significant neuroprotective activity, nearly completely suppressing perirhinal and piriform cortex lesion development.

Propofol offers an intriguing spectrum of pharmacological properties including $GABA_A$ agonist activity, NMDA antagonism (Orser et al., 1995; Ahmad and Pleuvry, 1995) and antioxidant activity (Murphy et al., 1992; Murphy et al., 1993; Young et al., 1997). This is a critical difference from other $GABA_A$ agonists such as diazepam or midazolam given the predominate role of glutamate and NMDA receptors in both Li-pilo and organophosphorus nerve agent mediated SE (Ormandy et al., 1989; Walton and Treiman, 1991; McDonough and Shih, 1997). The SE-induced NMDA receptor activation leads to an increased production of reactive oxygen species (Lafon-Cazal et al., 1993; Coyle and Puttfarcken, 1993; Michaelis, 1998) that are hypothesized to mediate the neuropathology induced by SE (Bruce and Baudry, 1995; Rong and Baudry, 1996; Rong et al., 1999; Peterson et al., 2002 in Appendix). We propose that propofol's NMDA antagonism would attenuate the NMDA receptor activation while the antioxidant activity suppresses the activity of the reactive oxygen species. These properties offer significant advantages of propofol over midazolam as a medical countermeasure for nerve agent exposure.

The apparent combination of anticonvulsant and neuroprotectant activity combined with commercial availability and existing FDA approval qualifies propofol as a critical candidate for development as a first line medical countermeasure for OP nerve agent exposure. The PI's laboratory and collaborators are in a position to continue propofol testing including the development of intravenous administration strategies. This would be combined with assessments of neuropathology and behavioral effects as well as an evaluation of the SE-mediated induction of specific apoptotic and necrotic gene markers.

The production of reactive oxygen species and in particular superoxide in Li-pilo convulsions has been characterized by a study performed in this laboratory (Peterson et al., 2002 in Appendix). The fluorescent dye hydroethidine was used as a marker of reactive oxygen species production, most notably superoxide, during Li-pilo-induced SE. Enhanced superoxide production was observed in regions damaged by Li-pilo SE. Using the hydroethidine technique it would be possible to test a hypothesis that the antioxidant activity of propofol reduces superoxide production during SE. Additional experiments with the hydroethidine model might involve a longitudinal study of superoxide production in critical brain regions during the 24 hour course of SE progression.

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APPENDIX

1. **Abstract Reprint:** Peterson, S.L., Purvis, R.S. and Griffith, J.W., (2002). Propofol Inhibition of Lithium-Pilocarpine-Induced Status Epilepticus. Epilepsia 43 (S7): 19.

2. **Publication Reprint:** Peterson, S.L., Morrow, D., Liu, S. and Liu, K.J., (2002). Hydroethidine Detection of Superoxide Production During Lithium-Pilocarpine Model of Status Epilepticus. Epilepsy Research 49:226-238.

PROPOFOL INHIBITION OF LITHIUM-PILOCARPINE–INDUCED STATUS EPILEPTICUS

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Rationale: The status epilepticus (SE) induced by lithium–pilocarpine (Li-pilo) treatment in rats produces neuropathology similar to that of the organophosphrous (OP) nerve agents. Because ongoing cholinergic convulsions are difficult to arrest with current treatments, this study was designed to determine the efficacy of the nonbarbiturate anesthetic propofol against Li-pilo–induced SE. **Methods:** Anesthetized Sprague–Dawley rats were implanted with electrocorticographic (EcoG) electrodes. After 7–10 days of recovery, they were administered 3 mmol/kg LiCl, s.c., followed 20–24 h later by 25 mg/kg pilocarpine, s.c. Propofol was administered i.p. either immediately after pilocarpine exposure, after 5 min of SE, or after 3 h of SE, as defined by continuous, high-amplitude ECoG activity. Animals were killed 24 h after pilocarpine, and the brains sectioned for hematoxylin and eosin (H&E) stain. **Results:** All animals survived the 24-h period after 3 h of SE when treated with 55 mg/kg propofol, but only half (three of six) survived after 50 mg/kg propofol. All subsequent experiments tested 55 mg/kg propofol. Propofol prevented SE onset after pilocarpine exposure and terminated all seizure activity when administered during SE. The latency to inhibit SE was longer after 3-h SE than 5-min SE (20.8 vs. 12.8 min; *t* test, $p < 0.05$). Rats experiencing 3-h SE had substantial neuropathology in the perirhinal and especially the piriform cortex, with all animals demonstrating >40% necrotic or malacic tissue in that area. Significantly less neuropathology was found in the perirhinal and piriform cortex of rats treated with propofol after 5 min of SE as determined by histopathology rating (Mann–Whitney *U* test, $p < 0.025$) and optical density measurements (*t* test, $p < 0.01$) of H&E-stained sections. **Conclusions:** This study is the first to demonstrate that propofol effectively terminates ongoing Li-pilo–induced SE and decreases neuropathology associated with those seizures. Propofol may serve as effective treatment of OP nerve exposure. (Supported by Department of Army award no. DAMD 17-01-1-0794. The US Army Medical Research Acquisition Activiy, 820 Chandler Street, Fort Detrick, MD 21702-5014 is the awarding and administering acquisition office.)

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Hydroethidine detection of superoxide production during the lithium–pilocarpine model of status epilepticus

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Abstract

Hydroethidine is reported to be selectively oxidized to ethidium by superoxide. Using digital imaging and fluorescence microscopy it is possible to evaluate neuronal ethidium accumulation in specific brain regions of rats damaged in the lithium–pilocarpine model of status epilepticus. Intravenous or intraperitoneal administration of hydroethidine prior to 1 h of status epilepticus produced diffuse cytosolic distribution of ethidium fluorescence suggesting an increased neuronal production of superoxide that was not observed in control animals. A significantly increased number of neurons with the enhanced ethidium fluorescence was observed in parietal cortex, piriform cortex, perirhinal cortex, lateral amygdala, mediodorsal thalamus and laterodorsal thalamus, suggesting superoxide as a mechanism of neuronal injury in those regions. Other regions injured by lithium-pilocarpine seizures, such as the basolateral amygdala and hippocampus, did not demonstrate the enhanced neuronal ethidium fluorescence. In such regions it is possible that superoxide is not a mechanism of injury or that 1 h of status epilepticus is not sufficient to produce superoxide or other reactive oxygen species. © 2002 Elsevier Science B.V. All rights reserved.

Keywords: Hydroethidine; Ethidium; Superoxide; Lithium; Pilocarpine; Status epilepticus

1. Introduction

Pilocarpine administration 20–24 h following lithium chloride treatment produces status epilepticus (SE) of several hours duration (Honchar et al., 1983; Clifford et al., 1987). Following a latent period of several weeks the animals that experienced the SE develop spontaneous limbic seizures that increase in frequency (Dubé et al., 2000, 2001). In addition, lithium–pilocarpine (Li-pilo)-induced SE results in well-characterized neuropathology in cortex, thalamus, hippocampus and amygdala regions (Honchar et al., 1983; Clifford et al., 1987; Motte et al., 1998; Peredery et al., 2000; Dubé et al., 2000, 2001), without which spontaneous seizures do not develop (Lemos and Cavalheiro, 1995).

The mechanism of Li-pilo-induced SE and the resultant neuronal damage are unclear. Following an initial cholinergic phase of the toxicity a distinct noncholinergic phase occurs in which exces-

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sive glutamate and aspartate release induces SE and the associated neuropathology by excitation of excitatory amino acid receptors (Clifford et al., 1987; Ormandy et al., 1989; Walton and Treiman, 1991; McDonough and Shih., 1997; Solberg and Belkin, 1997). *N*-methyl-D-aspartic acid (NMDA) receptors are crucial to the seizure mechanism as the SE induced by Li-pilo is inhibited by selective NMDA antagonists (Walton and Treiman, 1991; Ormandy et al., 1989). The intracellular influx of Ca^{++} associated with NMDA excitotoxicity induces a multitude of signal transduction mechanisms that contribute to the metabolic stress of mitochondria (Michaelis, 1998). The NMDA receptor-mediated metabolic stress leads to excessive oxidative phosphorylation and the increased production of reactive oxygen species (ROS) such as superoxide, hydrogen peroxide and hydroxyl radicals (Lafon-Cazal et al., 1993; Coyle and Puttfarcken, 1993; Michaelis, 1998). Superoxide may also react with nitric oxide to produce the strong oxidizing agent peroxynitrite (Michaelis, 1998).

There is increasing evidence that ROS are responsible for the neuropathology induced by SE (Bruce and Baudry, 1995; Rong and Baudry, 1996; Rong et al., 1999). In that regard, kainic acid (KA)-induced SE activates transcription factors such as nuclear factor κB (NFκB) and activator protein-1 (AP-1) which are known to be activated by oxidative stress (Rong and Baudry, 1996; Rong et al., 1999). SE also activates the ROS scavenging enzymes superoxide dismutase (SOD) and catalase indicating a cellular response to increased ROS (Bruce and Baudry, 1995; Ferrer et al., 2000). SE induces ROS mediated protein oxidation as measured by tyrosine nitration (Bruce and Baudry, 1995; Rong et al., 1999) as well as lipid peroxidation as indicated by malondialdehyde (Bruce and Baudry, 1995). Antioxidants and free radical spin trap agents that react with ROS before they induce damage have been shown to reduce the neuropathology associated with SE (Armstead et al., 1989; Baran et al., 1994; Schulz et al., 1995; MacGregor et al., 1996; Rong et al., 1999). These accumulated data support a hypothesis that ROS play a critical role in the neuropathology induced by SE.

Hydroethidine (HEt) is a fluorescent dye that is oxidized to ethidium (ET) to a greater extent by superoxide than other ROS (Bindokas et al., 1996; Kondo et al., 1997; Buxser et al., 1999). ET fluoresces at a different wavelength (Ex = 495 nm; Em = 595 nm) than HEt (Ex = 365 nm, Em = 415 nm) and thus may be used to visualize superoxide production. HEt oxidation to ET has been used to characterize superoxide production in experimental models of ischemic stroke (Chan et al., 1998; Murakami et al., 1998), in response to NMDA receptor activation (Bindokas et al., 1996) and other in vivo models of pathology (Lewen et al., 2001).

The present study was designed to test the hypothesis that superoxide is produced in the brain during Li-pilo-induced SE. The oxidation of HEt to ET was used to characterize directly superoxide production in specific brain regions during the first hour of Li-pilo SE. If superoxide is a mechanism of neuropathology in these seizures, then an enhanced accumulation of ET was expected in those brain regions damaged by Li-pilo SE.

2. Methods

2.1. Animals

Male, Wistar rats obtained from Charles River (Wilmington, MA) and weighing 350–400 g at the time of seizure test were used for these experiments. The animals were maintained in a climate-controlled vivarium at 21 °C on a 12-h light:12-h dark cycle and allowed free access to food and water. All the animal care and use conformed to the policies of the University of New Mexico Health Sciences Center.

2.2. Intracranial implants

The rats were anesthetized with equithesin (a mixture of chloral hydrate, pentobarbital, magnesium sulfate, ethanol, propylene glycol and water) for the surgical implantation of the electrocorticogram (ECoG) recording electrodes. Stainless steel screws were placed bilaterally in the skull 3 mm

lateral to midline and equidistant between bregma and lambda. The screws were attached to connector pins by insulated wire. A third screw assembly was placed over the frontal sinus as a reference electrode. Additional screws were set in the skull to serve as anchors. All connector pins were inserted into a McIntyre connector (Ginder Scientific, Ottawa, ON). Screws, wires and connectors were secured in place with dental acrylic cement and the incision site closed with surgical staples. Postoperative antibiotics (25 000 IU Durapen) and analgesics (0.02 mg/kg buprenorphine) were administered. Animals were allowed seven to ten days recovery before seizure testing.

2.3. Seizure induction and ECoG recording

The day prior to the seizure induction the rats were administered SC 3 mmol/kg lithium chloride (Sigma, St. Louis, MO). The lithium administration always preceded the pilocarpine administration by 20–24 h. The following day the animals were placed in a seizure observation cage and connected to a Grass Model 8 electroencephalograph by way of the implanted McIntyre connector for recording of ECoG. Pilocarpine was administered SC in a dose of 20 mg/kg following 10 min of baseline ECoG recording. ECoG activity was recorded continuously throughout the experiment.

SE was defined as the occurrence of continuous high amplitude ECoG spiking (Ormandy et al., 1989). HEt was administered either IP or IV at the onset of SE. Following HEt administration the SE was allowed to continue for 1 h at which time the animals were anestheitized with equithesin for perfusion–fixation.

2.4. HEt administration

Rats were administered 0.5 mg HEt by either IP or IV administration. HEt was administered in a concentration of 1 mg/ml in 0.1 M phosphate buffer solution (PBS) containing 20% DMSO.

Placement of a femoral catheter was required for the IV administration experiment. On the day of seizure testing the rats were anesthetized with 3% halothane in 70% nitrous oxide and 30% oxygen for anesthesia induction. Halothane was reduced to 1.2% for anesthesia maintenance. A femoral catheter was placed and externalized through the closed incision. Following the surgery, the rats were placed in the seizure observation cage and allowed a minimum of 30 min recovery from the halothane anesthetic before pilocarpine was administered. The 0.5 mg HEt was administered IV at the onset of Li-pilo-induced SE.

2.5. In situ detection of ROS production

All animals were sacrificed by intraaortic perfusion-fixation while anesthetized with equithesin. The animals were initially perfused with heparinized PBS (12.5 IU/ml, Sigma) followed by 10% formalin PBS (VWR Scientific Products). Brains were removed and immersed in 10% formalin for a minimum of 24 h of postfixation. Following postfixation the brains were rinsed thoroughly with PBS and sectioned into 50 μm sections by a vibratome. Brain sections were treated with 0.2% (w/v) sodium borohydride (Sigma) in PBS for 20 min under a ventilation hood and transferred into PBS for rinsing (Clancy and Cauller, 1998). Sections were mounted on glass slides with a coverslip using Gel/Mount mounting media (Fisher Scientific). Tissue sections were viewed using a microscope (Olympus BH2-RFCA) equipped with epifluorescence optics and fluorescence viewed using filter settings of DAPI (360 nm excitation, 400 nm dichromic, 460 nm emission), FITC (480 nm excitation, 505 nm dichromic, 535 nm emission) and TRITC (545 nm excitation, 570 nm dichromic, 610 nm emission). Digital images were acquired with a digital camera (Olympus MLH 020550) using the Olympus MagnaFire Camera Imaging and Control software (Version 1.1) and analyzed using Image Pro Plus software (Version 4.1).

2.6. Data and statistical analysis

As described below, neurons were differentiated by the appearance of diffuse cytosolic ET fluorescence so that the experimental data could by analyzed in a semi-quantitative manner. Neurons

were considered positive for diffuse cytosolic fluorescence when the entire body of the soma was made visible by the accumulate ET fluorescence while the nucleus was obscured. The numbers of such cells in comparable regions of representative brain sections were counted by a single observer (SP) in a blinded fashion. The average number of neurons in corresponding regions that demonstrated diffuse cytosolic ET fluorescence were compared with Student's t-test (two tailed, $\alpha = 0.05$).

3. Results

3.1. Li-pilo SE

Pilocarpine administration in animals pretreated 20–24 h earlier with LiCl induced SE with an average latency of 33 min (range 19–60 min). HEt was administered either IV or IP within 5 min of the onset of SE as defined by continuous high frequency ECoG activity (Ormandy et al., 1989). All seizure animals treated with HEt completed 1 h of SE before sacrifice.

3.2. Morphological characteristics of ET fluorescence

Superoxide production was determined by oxidation of HEt to ET. Treatment of the tissue sections with sodium borohydrate significantly reduced background autofluorescence induced by the formalin fixative and allowed clear visualization of ET fluorescence (Clancy and Cauller, 1998). As previously reported under normal physiological conditions ET fluorescence appears as small particles in the cytosol, suggesting mitochondrial generation of superoxide (Kondo et al., 1997; Murakami et al., 1998; Chan et al., 1998). In the present experiment, such neurons were clearly visible in the piriform cortex (Fig. 1A and B) and parietal cortex (Fig. 2A and B) of seizure-control (no SE) animals treated with IV HEt. In rats that experienced 1 h of Li-pilo-induced SE following IV HEt, the ET fluorescent signal filled the cytosolic space and allowed clear visualization and differentiation of individual soma and dendritic processes. Such neurons are visible under higher magnification in the piriform cortex (Fig. 1C and D) and parietal cortex (Fig. 2C and D) in animals experiencing SE. Identical diffuse cytosolic ET fluorescence has been reported in ischemic neurons in experimental models of stroke (Kondo et al., 1997; Chan et al., 1998; Murakami et al., 1998). Neurons in which the accumulated fluorescence demonstrated the entire body of the soma and obscured the nucleus were considered positive for diffuse cytosolic ET fluorescence.

3.3. Regional distribution of ET fluoresence

HEt administered at the onset of 1 h of Li-pilo-induced SE produced a differential regional distribution of superoxide generation as determined by HEt oxidation to ET (Table 1). In cortical regions, a significantly greater number of neurons exhibited diffuse cytosolic ET fluorescence in piriform layer III (Fig. 1, $P < 0.01$), parietal cortex (Fig. 2, $P < 0.01$), perirhinal cortex (Fig. 3A, $P < 0.05$) and endopiriform nucleus (Fig. 3A, $P < 0.01$) in SE rats as compared with the control group.

Li-pilo-induced SE produced significant increases in the number of neurons with diffuse cytosolic ET fluorescence in thalamic regions (Figs. 4 and 3B). A significantly greater number of ET fluorescent neurons were identified in the laterodorsal and mediodorsal nuclei of the thalamus in SE rats (Fig. 4, $P < 0.01$).

Few differences in the number of ET fluorescent neurons were found in the amygdala regions (Table 1; Fig. 5). There were no significant differences in basomedial or basolateral amygdaloid nuclei between the seizure-control and SE groups (Fig. 5) although a significantly greater number of neurons were identified in the lateral amygdaloid nucleus of rats with SE (Table 1, $P < 0.01$). A noticeable degree of background fluorescence was found in the amygdala of seizure-control rats that received no HEt (Fig. 5). Also, seizure-control (no SE) animals treated with IV HEt exhibited a number of neurons with diffuse cytosolic fluorescence in the basomedial and basolateral nuclei (Fig. 5). While both the seizure-control (no SE)

and SE rats demonstrated more hippocampal fluorescence than untreated rats (No HEt) differences between the seizure-control (no SE) and SE groups were not distinguishable (Fig. 5).

No significant differences in the number of ET fluorescent neurons were found in hippocampal CA1, CA2, CA3 or dentate gyrus regions (Table 1, Fig. 5). As indicated for the amygdala, there was a noticeable degree of neuronal fluorescence in hippocampal neurons of seizure-control rats that received no HEt. Further, hippocampal neurons in SE rats treated with IV HEt did not exhibit the diffuse cytosolic ET fluorescence pattern observed in neurons from other brain regions. While both seizure-control (no SE) and SE rats demonstrated more hippocampal fluorescence than untreated rats (No HEt) differences between the seizure-control (no SE) and SE groups were not distinguishable (Fig. 5).

3.4. IP vs. IV HEt administration

IP HEt administration at the onset of 1 h of Li-pilo-induced SE produced neuronal ET fluorescent signals that were not as intense or readily differentiated as those produced by IV administration. Regardless, neurons with diffuse cytosolic ET fluorescence were discernable and a regional neuron count was employed to determine superoxide production.

The results of IP HEt administration were identical to those reported following IV administration with the exception of the endopiriform nucleus where the difference was not statistically

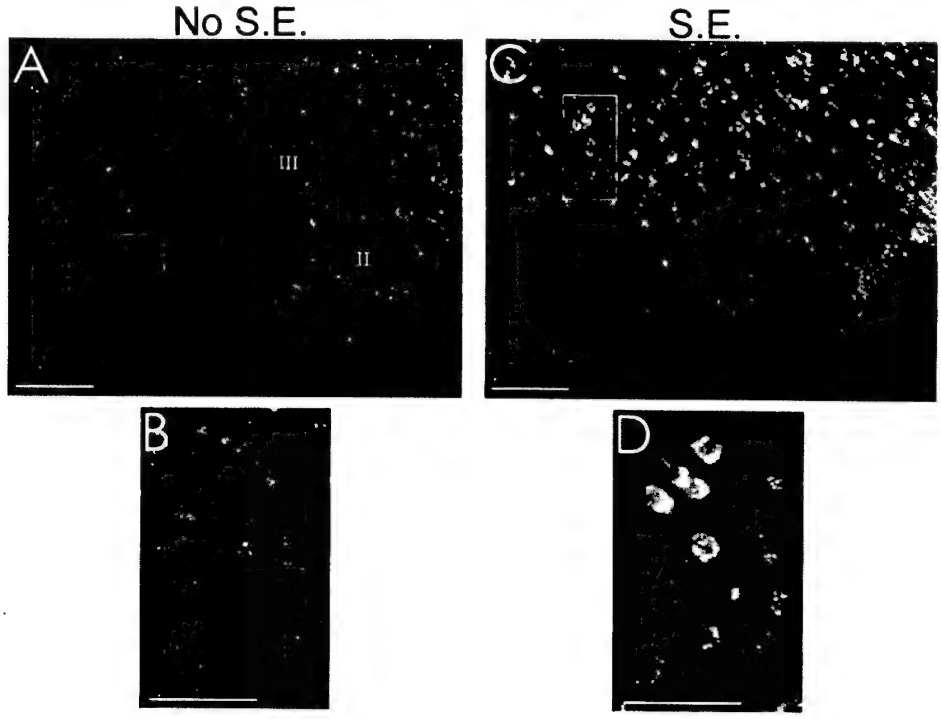

Fig. 1. Representative photomicrographs of superoxide production as determined by oxidation of HEt in rat piriform cortex. HEt was administered IV 5 min following the onset of SE and 1 h prior to sacrifice. (A) Basal level of ET fluorescence in seizure-control rat that was administered HEt but did not undergo SE (no S.E.). ET fluorescence appears as small particles in the cytosol, suggesting mitochondrial generation of superoxide under normal physiological conditions. (B) Magnification of insert shown in panel A. (C) ET fluorescence in piriform cortex following 1 h of Li-pilo-induced SE (S.E.). ET fluorescence signal fills the cytosolic space and allows visualization of soma. In many neurons the accumulated fluorescence differentiates the entire body of the soma and obscures the nucleus. (D) Magnification of insert shown in panel C. Scale bars 100 μm in A, C; 50 μm in B, D. II: piriform cortex layer II; III: piriform cortex layer III.

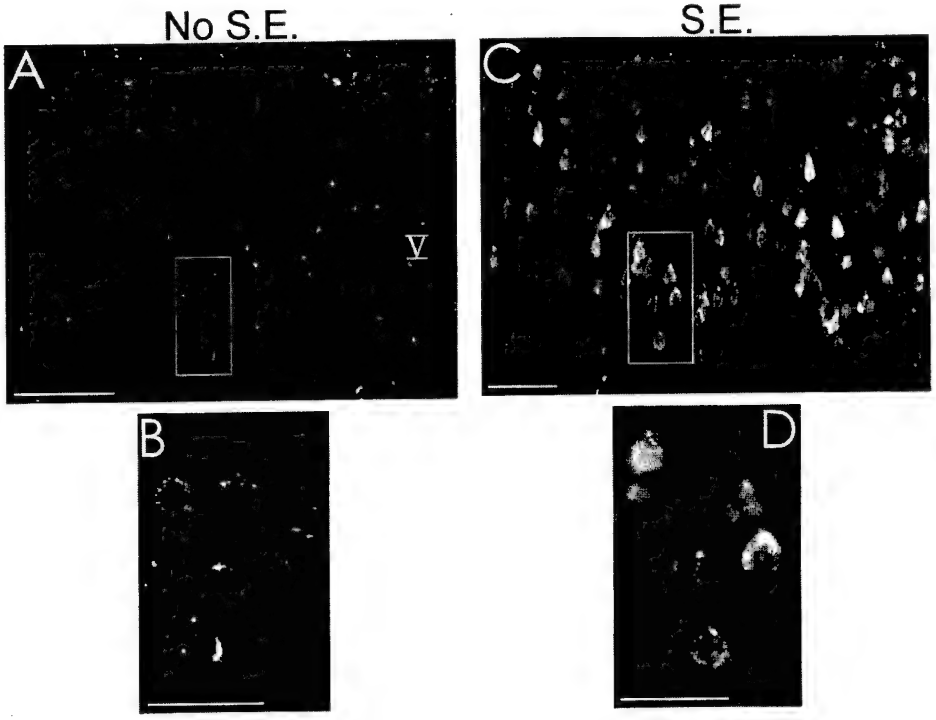

Fig. 2. Representative photomicrographs of superoxide production in parietal cortex following IV HEt administration. (A) Particle-like ET fluorescence in seizure-control rat that was administered HEt but did not undergo SE. (B) Magnification of insert shown in panel. (C) Diffuse cytosolic ET fluorescence pattern in parietal cortex neurons following SE. (D) Magnification of insert in panel C. Scale bars: 100 μm in A, C; 50 μm in B, D. V: parietal cortex layer V.

significant (Table 2). A significantly greater number of neurons exhibiting diffuse cytosolic ET labeling were found in parietal cortex, layer III of piriform cortex, perirhinal cortex, mediodorsal thalamus, laterodorsal thalamus, lateral amygdala (Table 2). Distribution of perirhinal cortex neurons with diffuse cystolic ET labeling following IP HEt administration is shown in Fig. 6.

4. Discussion

The primary finding of this experiment was that superoxide is produced in regions of the brain damaged by Li-pilo-induced SE. To our knowledge, this represents the first direct demonstration of ROS production in this important model of cholinergic convulsions and epileptogenesis. Using the oxidation of HEt as a marker of superoxide production, enhanced accumulation of ET fluorescence was observed in the parietal cortex, piriform cortex layer III, perirhinal cortex, endopiriform nucleus, lateral amygdala, mediodorsal thalamus and laterodorsal thalamus. This may indicate that superoxide production is a mechanism of neuronal damage in those regions. Regions without significant ET accumulation included piriform cortex layer II, basomedial amygdala, basolateral amygdala and the hippocampus. Although Li-pilo SE damages these regions, the present data indicate that either the mechanism of neuronal damage in these regions does not involve superoxide or that superoxide is not produced during the first hour of SE in these regions.

Previous studies have demonstrated superoxide production in other experimental models of epilepsy. Reduction of the SOD-inhibitable nitroblue tetrazolium was used to demonstrate cerebral superoxide production during bicuculline-induced seizures in newborn pigs

(Armstead et al., 1989). Changes in dihydrorhodamine 123 fluorescence were used to detect superoxide production in rat brain slices during epileptiform activity (Frantseva et al., 2000). Using aconitase inactivation as a marker, superoxide was shown to be increased at times preceding neuronal death in KA-induced seizures in mice (Liang et al., 2000). SOD activity was significantly increased in rats 5 days following KA administration, suggesting an adaptive response to superoxide production (Bruce and Baudry, 1995). The present results provide direct evidence for the formation of superoxide during Li-pilo-induced SE. Although SE and seizure models vary in neuronal mechanisms and the specific brain regions damaged, (Ben-Ari, 1985; McDonough et al., 1997; Goodman, 1998) the current results add to the accumulating evidence that superoxide is a product of ongoing seizure activity.

IP administration of HEt provided nearly identical results without the technical difficulty associated with IV administration. The diffuse, cytosolic ET fluorescence was not as clearly differentiated as the IV study but the reduction of background autofluorescence with sodium borohydrate improved the discrmination of the neuronal soma. It is hypothesized that fixative aldehydes react with tissue amines to form fluorescent Schiff's bases (Clancy and Cauller, 1998). Sodium borohydrate acts to reduce the amine-aldehyde compounds into non-fluorescent salts, thereby reversing background autofluorescence (Clancy and Cauller, 1998).

HEt is reported to be oxidized to ET selectively by superoxide (Buxser et al., 1999; Kondo et al., 1997). In an extensive series of in vitro, cell culture and brain slice assays, Bindokas et al. (1996) concluded that HEt is selectively oxidized by su-

Table 1
Number of neurons with diffuse cytosolic Et fluorescence in various brain regions following IV HEt administration

	Control ($n = 5$)	SE ($n = 6$)	Variation (%)
Cortex			
Parietal-layer V	17.0 ± 5.8	110.2 ± 18.5**	548
Piriform-layer II	5.2 ± 1.6	8.5 ± 2.7	63
Piriform-layer III	5.4 ± 0.8	63.3 ± 14.4**	1113
Perirhinal	32.2 ± 22.0	135.3 ± 26.9*	320
Endopiriform	1.8 ± 0.5	15.2 ± 2.3**	744
Thalamus			
Mediodorsal–central	2.0 ± 0.3	74.0 ± 14.0**	3600
Mediodorsal–medial	2.2 ± 0.5	48.8 ± 8.6**	2118
Mediodorsal–lateral	2.0 ± 0.3	30.7 ± 5.6**	1435
Lateraldorsal–medial	3.0 ± 0.8	61.0 ± 12.0**	1933
Lateraldorsal–lateral	5.4 ± 1.5	109.2 ± 21.5**	1922
Amygdala			
Basomedial	21.4 ± 2.2	22.0 ± 2.9	3
Basolateral–anterior	32.2 ± 10.5	40.0 ± 17.0	24
Basolateral–posterior	25.8 ± 13.5	34.3 ± 9.9	33
Lateral	8.4 ± 2.5	24.3 ± 3.0**	189
Hippocampus			
CA_1	1.6 ± 0.4	1.7 ± 0.2	6
CA_2	1.8 ± 0.5	1.8 ± 0.3	0
CA_3	1.8 ± 0.6	1.7 ± 0.2	−5
Dentate grus-granular	1.2 ± 0.2	1.2 ± 0.2	0
Dentate gyrus-polymorphic	1.6 ± 0.6	1.3 ± 0.2	−19

SE indicates 1 h of Li-pilo-induced SE. Statistical significance determined by Student's t-test, *$P<0.05$, **$P<0.01$ as compared with control.

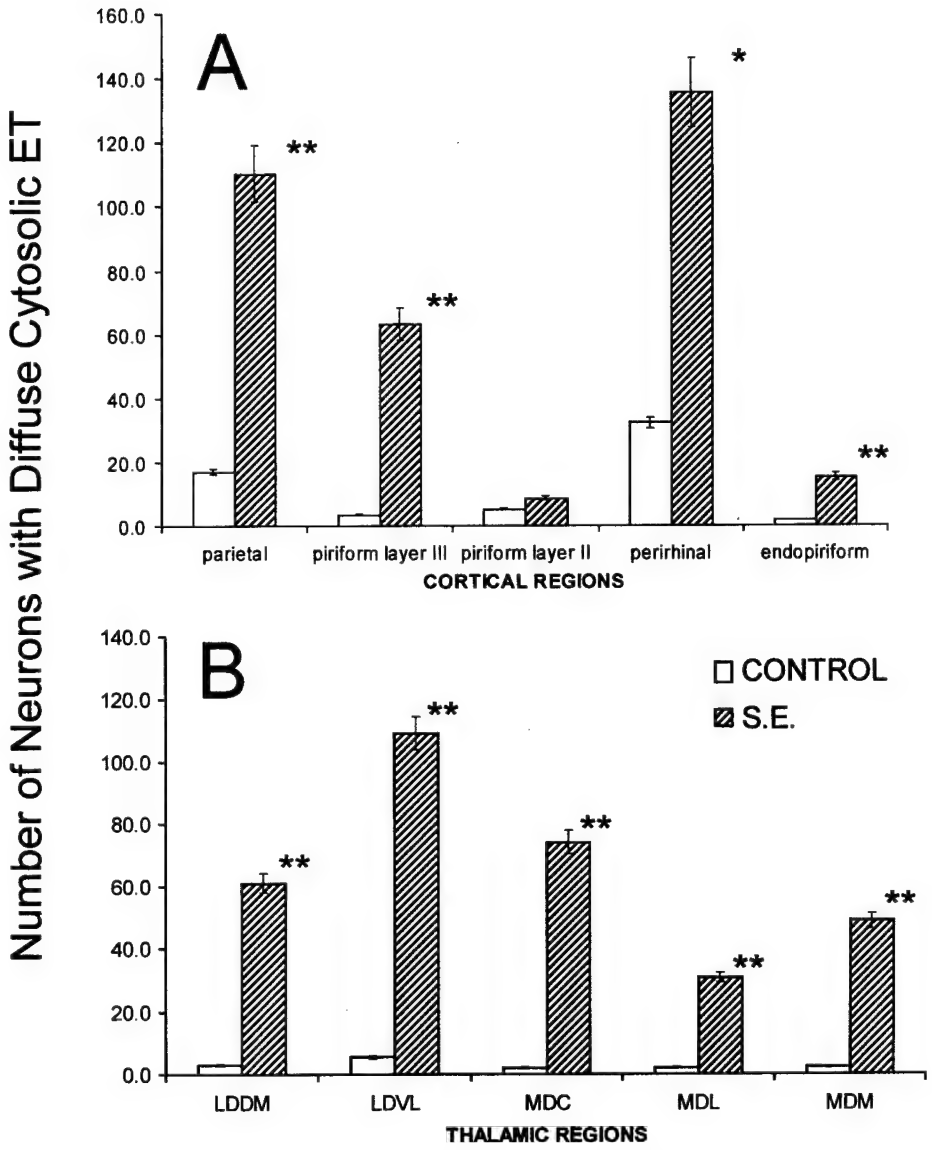

Fig. 3. Number of neurons with diffuse cytosolic ET fluorescence in cortical (A) and thalamic (B) brain regions. Solid bars indicate response of animals that experienced 1 h of Li-pilo-induced status epilepticus (SE). HEt was administered IV at onset of SE * indicates significant difference from corresponding seizure-control (no SE) group (Students t-test, $P < 0.05$), ** indicates $P < 0.01$.

peroxide. Since then, HEt has been used as a selective marker of superoxide production in a variety of in vivo (Lewen et al., 2001), isolated tissue (Miller et al., 1998; Zou et al., 2000) and cell culture (Schuchmann and Heinemann, 2000; Barbacanne et al., 2000) models of disease. In cerebral ischemia experiments, small particulate ET fluorescence was indicative of mitochondrial superoxide production in normal, nonischemic neurons (Chan et al., 1998; Murakami et al., 1998). Diffuse cytosolic neuronal labeling that allowed differentiation of the soma and dendrites was evidence of increased superoxide production during ischemia (Chan et al., 1998; Murakami et al.,

1998), an effect that was significantly enhanced in transgenic SOD knockout mice (Kondo et al., 1997; Murakami et al., 1998). Similar patterns of neuronal ET fluorescence were observed in the present experiment with particulate ET fluorescence in neurons of normal rats without SE and

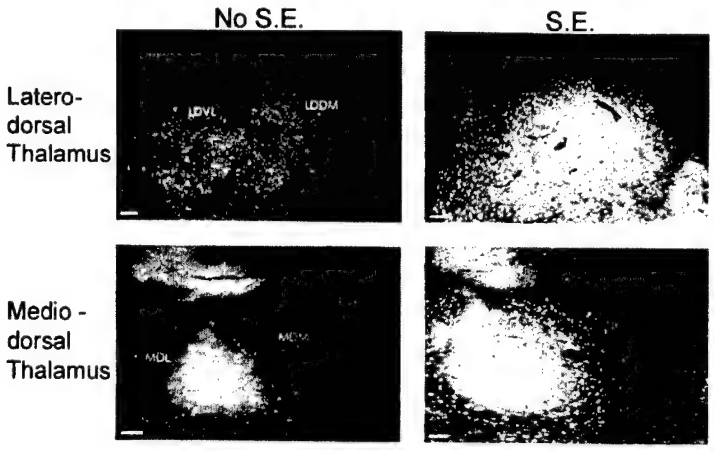

Fig. 4. ET accumulation in thalamic regions. Laterodorsal and mediodorsal thalamic nuclei contained little or no ET fluorescence in rats that did not undergo SE (No SE). In contrast, numerous neurons possessed diffuse cytosolic ET fluorescence in rats experiencing 1 h of Li-pilo-induced SE (SE). Scale bars are 100 μm. MDL: mediodorsal n. lateral, MDC: mediodorsal n. central, MDM: mediodorsal n. medial, LDDM: laterodorsal n. dorsomedial, LDVL: laterodorsal n. ventrolateral. HPC: hippocampus, PVP: paraventicular n. posterior.

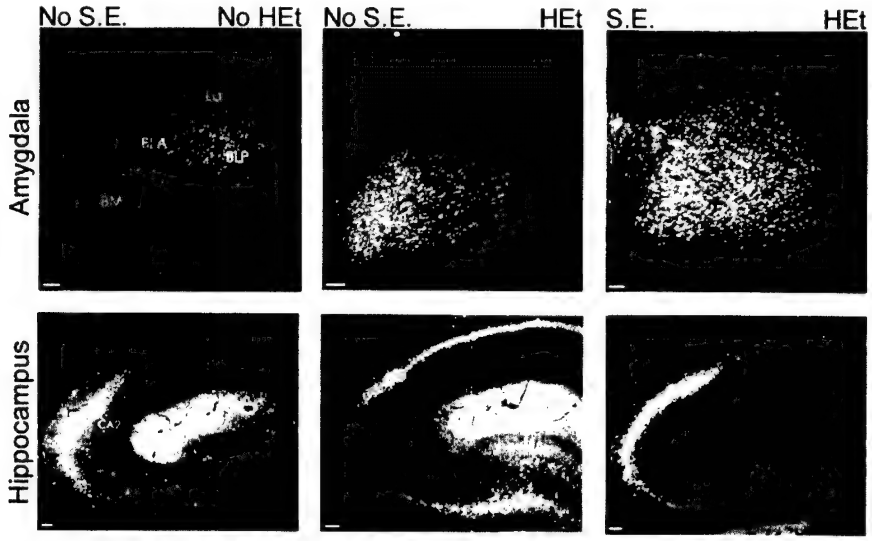

Fig. 5. ET accumulation in amygdala and hippocampus. Far left column represents seizure-control rats that received neither SE (No SE) nor HEt (No HEt). Background neuronal fluorescence was noted in these animals. The middle column represents seizure-control animals (No SE) that were administered HEt IV 1 h prior to sacrifice. The right column represents animals with SE (SE) and IV HEt (HEt) administration. Differences in the number of neurons with diffuse cytosolic ET fluorescence are difficult to discern in amygdala and hippocampal regions between animals with SE and those without. Scale bars are 100 μm. BM: basomedial amygdala; BLA: basolateral amygdala anterior; BLP: basolateral amygdala posterior; La: lateral amygdala; DEn: endopiriform nucleus; DG: dentate gyrus granular layer; PoDG: polymorphic layer dentate gyrus.

Table 2
Number of neurons with diffuse cytosolic Et fluorescence in various brain regions following IP HEt administration

	Control ($n=4$)	SE ($n=5$)	Variation (%)
Cortex			
Parietal-layer V	4.0 ± 2.7	84.0 ± 2.2**	2000
Piriform-layer II	2.0 ± 0.4	4.0 ± 1.1	100
Piriform-layer III	4.0 ± 0.9	38.0 ± 6.4**	850
Perirhinal	6.0 ± 2.1	70.0 ± 14.2**	1600
Endopiriform	1.5 ± 0.5	6.0 ± 2.1	300
Thalamus			
Mediodorsal–central	3.3 ± 0.8	44.2 ± 9.2**	1239
Mediodorsal–medial	2.0 ± 0.4	39.2 ± 6.3**	1860
Mediodorsal–lateral	1.5 ± 0.3	19.6 ± 5.1*	1207
Lateraldorsal–medial	2.0 ± 0.7	51.2 ± 12.1**	2460
Lateraldorsal–lateral	2.0 ± 0.7	40.8 ± 13.4*	1940
Amygdala			
Basomedial	1.0 ± 0.0	2.6 ± 6.7	160
Basolateral–anterior	4.0 ± 1.3	4.0 ± 0.9	0
Basolateral–posterior	2.5 ± 0.9	4.6 ± 0.9	84
Lateral	1.5 ± 0.3	3.2 ± 0.6*	113
Hippocampus			
CA_1	1.0 ± 0.0	1.2 ± 0.2	20
CA_2	1.0 ± 0.2	1.4 ± 0.3	40
CA_3	1.3 ± 0.2	1.0 ± 0.0	−23
Dentate gyrus-granular	0.5 ± 0.3	1.0 ± 0.3	50
Dentate gyrus-polymorphic	1.5 ± 0.5	1.8 ± 0.8	20

SE indicates 1 h of Li-pilo-induced SE. Statistical significance determined by student t-test, *$P<0.05$, **$P<0.01$ as compared with control.

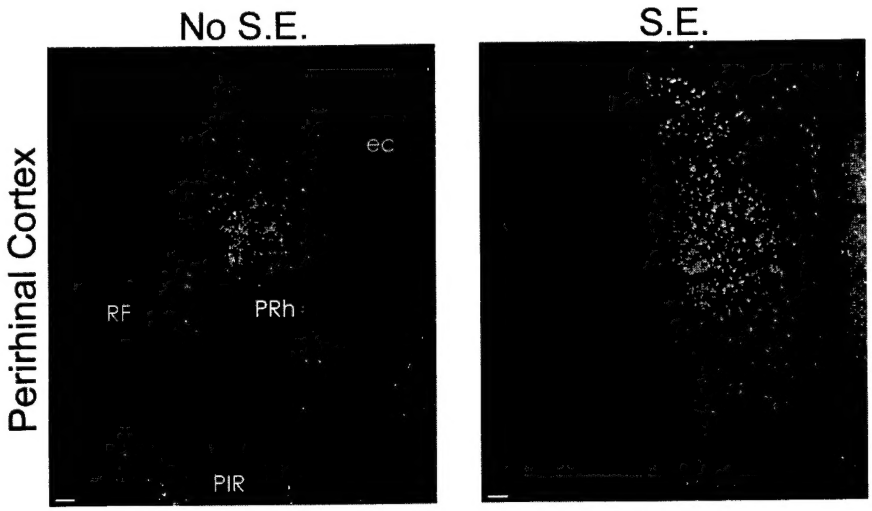

Fig. 6. ET accumulation in perirhinal cortex following IP HEt administration. Perirhinal neurons contained little or no ET fluorescence in seizure-control rats (no SE). Numerous neurons possessed diffuse cytosolic ET fluorescence in rats experiencing 1 h of Li-pilo-induced SE (SE). Scale bars are 100 μm. RF: rhinal fissure; PIR: piriform cortex; perirhinal cortex; ec: external capsule.

diffuse cytosolic fluorescence in neurons of SE rats suggesting that HEt may be used as a direct measure of SE-induced superoxide production in vivo. However, further experiments using pharmacological agents that selectively alter superoxide activity will be required to verify the selectivity of HEt as a marker of superoxide.

The hypothesis of this experiment was that excitatory amino acid-induced superoxide production contributes to the neuronal damage associated with Li-pilo SE (Armstead et al., 1989; Lafon-Cazal et al., 1993; Coyle and Puttfarcken, 1993; Vallett et al., 1997; Michaelis, 1998; Frantseva et al., 2000; Yasuda et al., 2001). As described below, numerous brain regions damaged by Li-pilo SE demonstrated enhanced accumulation of ET fluorescence and we propose that superoxide is responsible at least in part for the neuronal damage. However, several regions damaged by Li-pilo SE did not demonstrate significant increases in ET production in the present study. One explanation may be that ROS other than superoxide or that nonROS mechanisms mediate neuronal damage in these areas. Other ROS that are linked to seizure activity include nitric oxide (Yasuda et al., 2001) and hydroxyl radical (Vallett et al., 1997). Alternatively, the 1 h SE duration used in this study may not have been of sufficient duration to generate either superoxide or neuronal damage in these areas. Negligible neuronal damage occurs unless SE lasts at least 1 h in Li-pilo (Motte et al., 1998) or 40 min in pilocarpine seizures (Fujikawa, 1996; Lemos and Cavalheiro, 1995). The 1 h SE used in this study may not have been sufficient to induce significant superoxide production in all brain regions.

Severe neuropathology with almost complete cell loss following Li-pilo SE has been reported in piriform cortex (Honchar et al., 1983; Clifford et al., 1987; Motte et al., 1998; Peredery et al., 2000; Dubé et al., 2000, 2001) and the endopiriform nucleus (Peredery et al., 2000). Moderate cell loss was found in parietal cortex (Peredery et al., 2000; Dubé et al., 2000) and the deep layers of perirhinal cortex (Peredery et al., 2000). In the present study, increased ET accumulation was found in piriform cortex layer III and deep layers of the perirhinal cortex suggesting superoxide as possible mechanisms of pathology in those regions. No significant ET production was observed in piriform cortex layer II, suggesting either a delay in superoxide production or an alternative mechanism of pathology.

Several thalamic nuclei are damaged by Li-pilo SE (Honchar et al., 1983), particularly the mediodorsal (Clifford et al., 1987; Motte et al., 1998; Peredery et al., 2000; Dubé et al., 2000, 2001) and laterodorsal nuclei (Clifford et al., 1987; Peredery et al., 2000; Dubé et al., 2000, 2001). In the present study, some of the most obvious examples of diffuse cytosolic fluorescence were observed in these regions. This suggests an involvement of superoxide during the first hour of SE in the severe neuropathology-induced in these regions by Li-pilo SE.

Li-pilo SE induces consistently severe amygdala damage (Honchar et al., 1983; Clifford et al., 1987) particularly in the medial (Clifford et al., 1987; Peredery et al., 2000; Dubé et al., 2000, 2001), basolateral (Motte et al., 1998; Peredery et al., 2000; Dubé et al., 2000, 2001) and lateral nuclei (Clifford et al., 1987; Peredery et al., 2000). Our study found significant increases in ET only in the lateral amygdala nuclei. Interestingly, a number of neurons with diffuse cytosolic fluorescence was observed even in the seizure-control (no SE) rats treated with IV HEt (Fig. 5). This may indicate an ongoing superoxide production in the basomedial and basolateral nuclei under our experimental conditions that obscures seizure-induced superoxide production during 1 h of Li-pilo SE.

Damage with varying degrees of severity has been reported in CA1 and CA3 regions following Li-pilo SE (Honchar et al., 1983; Clifford et al., 1987; Motte et al., 1998; Peredery et al., 2000; Dubé et al., 2000, 2001). The dentate gyrus was frequently damaged (Clifford et al., 1987; Peredery et al., 2000) particularly the polymorphic or hilus regions (Motte et al., 1998; Clifford et al., 1987; Dubé et al., 2000, 2001). In spite of such reports, no significant differences in ET fluorescence were detected in any hippocampal region. As noted in the amygdala, a high degree of ET fluorescence was found in the seizure-control animals (Fig. 5). The nuclei of CA1 and CA3 neurons in SE rats were clearly discernable indicating a lack of diffuse cytosolic fluorescence observed in other brain regions. This suggests that little super-

oxide was produced in the hippocampus during the 1 h of Li-pilo SE.

Using HEt as an in situ imaging method for superoxide radical production, the present study provides evidence that superoxide is produced during the first hour of Li-pilo-induced SE in rats. The detection of ET in brain regions damaged by Li-pilo SE would indicate that superoxide is a mechanism of neural injury in these regions. Failure to detect significant ET production in regions damaged by Li-pilo SE indicate neural injury by mechanisms other than superoxide or a delay in superoxide production. These results suggest that drugs that act as SOD-mimetics may serve as neuroprotectants during the course of SE that is refractory to anticonvulsant therapy.

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